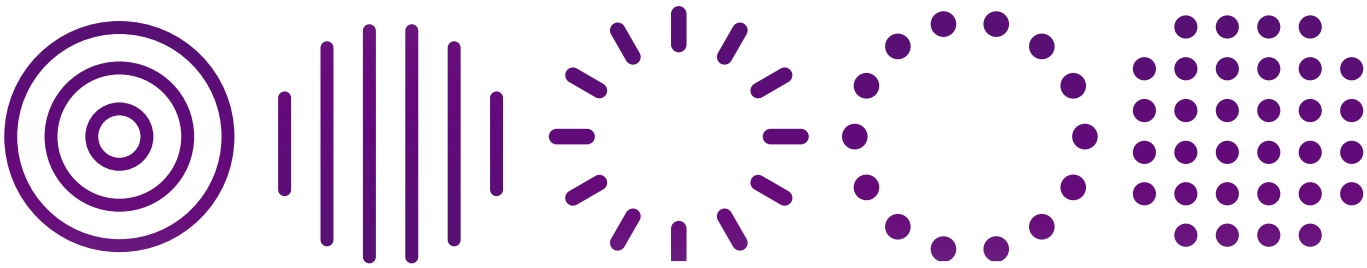
STOmics

Stereo-seq TRANSCRIPTOMICS SET FOR Stereo-seq Large Chip Designs (≤ 2cm * 3cm)

USER MANUAL

COMPATIBLE WITH 1cm * 2cm, 2cm * 2cm, and 2cm * 3cm chips.



Cat. No.:

111ST122 Stereo-seq Transcriptomics Set (1 cm * 2 cm), 2RXN
111ST221 Stereo-seq Transcriptomics Set (2 cm * 2 cm), 1RXN
111ST231 Stereo-seq Transcriptomics Set (2 cm * 3 cm), 1RXN

Kit Version: V1.0

Manual Version: A_1

REVISION HISTORY

Manual Version: A
Kit Version: V1.0
Date: Aug. 2023
Description:

- Initial release

Manual Version: A_1
Kit Version: V1.0
Date: Sep. 2023
Description:

- Error fixation

Note: Please download the latest version of the manual and use it with the corresponding Stereo-seq Transcriptomics T kit.

WORKFLOW

TOTAL TIME: ~1.5 DAYS

TABLE OF CONTENTS

NOTE: Additional operation tips and guidance.

CRITICAL STEPS: Pay extra attention for these steps to avoid experimental setbacks or problematic results.

QUALITY CHECK POINT

CAUTION: Proceed with extra care; improper handling or carelessness may cause experimental failure or accidents.

STOP POINT: Here you may pause your experiment and store your sample.

CHAPTER 1
INTRODUCTION

1.1. Intended Use

STOmics Stereo-seq Transcriptomics Set for Chip-on-a-slide is intended for generating a spatially-resolved 3' mRNA library from biological tissue sections. Built upon DNA Nanoball (DNB) technology, STOmics Stereo-seq Transcriptomics Set for Chip-on-a-slide enables a "tissue-to-data" solution through in situ capture of the whole transcriptome, at nanoscale resolution and centimeter-sized field of view. This kit utilizes DNB patterned array chips loaded with spatially-barcoded probes that capture and prime poly-adenylated mRNA from tissue sections in situ. Each cDNA synthesized from mRNA captured on a particular spot is linked to its spatially-barcoded probe, allowing subsequent gene expression mapping of a tissue section following sequencing and visualization analysis using the StereoMap visualization platform.

All reagents provided within this kit have passed stringent quality control and functional verification, ensuring performance stability and reproducibility.

1.2. Sequencing Guideline

Sequencing libraries produced via the Stereo-seq Transcriptomics Set for Chip-on-a-slide requires the DNBSEQ sequencing platform. For details, please refer to **Chapter 5: Library Construct and Sequencing** of this manual.

1.3. List of Kit Components

Each Stereo-seq Transcriptomics Set for 1cm * 2cm (111ST122) consists of:

- 111KT002 Stereo-seq Transcriptomics T Kit, 2RXN *1 (2 RXN)
- 110CT122 Stereo-seq Chip T (1cm * 2cm) *2 (1 EA)

Each Stereo-seq Transcriptomics Set for 2cm * 2cm (111ST221) consists of:

- 111KT001 Stereo-seq Transcriptomics T Kit, 1RXN *1 (1 RXN)
- 110CT221 Stereo-seq Chip T (2cm * 2cm) *1 (1 EA)

Each Stereo-seq Transcriptomics Set for 2cm * 3cm (111ST231) consists of:

- 111KT001 Stereo-seq Transcriptomics T Kit, 1RXN *1 (1 RXN)
- 110CT231 Stereo-seq Chip T (2cm * 3cm) *1 (1 EA)

Stereo-seq Library Preparation Kit is not included in the Stereo-seq Transcriptomics Set for Large Chip Designs and needs to be purchased separately. If you wish to construct Stereo-seq library in-house, please refer to **Chapter 4: Library Preparation** for more detail.

Further information on catalog numbers, kit components and specifications are listed below (next page).

Upon receiving the Stereo-seq Chip T (1cm*2cm), Stereo-seq Chip T (2cm*2cm), or Stereo-seq Chip T (2cm*3cm), please follow the instructions in Stereo-seq Large Chip Designs (≤ 2cm * 3cm) Operation Guide For Receiving, Handling And Storing to properly store unopened Stereo-seq Chip T.

Performance of products may only be guaranteed before their expiration date. Proper performance is also subject to the products being transported, stored, and used in appropriate conditions.

Table 1-1

Stereo-seq Transcriptomics T Kit, 2RXN	**Cat. No.:111KT002**		
Stereo-seq Transcriptomics T Kit, 1RXN	**Cat. No.:111KT001**		
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
RI	1000028499		300 μL × 2
PR Enzyme	1000028500		10 mg × 1
PR Rinse Buffer	1000042897		880 μL × 1
Glycerol	1000031615		50 μL × 1
RT Reagent	1000028507	(transparent)	360 μL × 1
RT Oligo	1000028508	(transparent)	1 OD × 1
RT Additive	1000028502	(transparent)	44 μL × 1
ReverseT Enzyme	1000028509	(transparent)	22 μL × 1
TR Buffe	1000039987	(transparent)	3458 μL × 1
cDNA Release Enzyme	1000039986		182 μL × 5
cDNA Release Buffer	1000039988	(transparent)	3458 μL × 1
cDNA Primer	1000039989		72 μL × 1
cDNA Amplification Mix	1000040200		440 μL × 1
Storage Temperature: -25°C~-18°C		Transported by cold chain	Expiration Date: refer to label

Table 1-2

Kit	Component	Quantity
Stereo-seq Chip T (1cm * 2cm) Cat. No.: 110CT122	Stereo-seq Chip T (1cm * 2cm)	2 EA
Stereo-seq Chip T (2cm * 2cm) Cat. No.: 110CT221	Stereo-seq Chip T (2cm * 2cm)	1 EA
Stereo-seq Chip T (2cm * 3cm) Cat. No.: 110CT231	Stereo-seq Chip T (2cm * 3cm)	1 EA
Storage Temperature: -25°C~-18°C	Transported by cold chain	Expiration Date: refer to label

1.4. Additional Equipment and Materials

Table below lists equipment and materials needed for this protocol. Some common laboratory equipments not named in Table 1-3 are expected to be accessible by the user, for instance, ice maker, biological safety cabinet, freezers, etc. For specific microscope requirements, please refer to **STOmics Microscope Assessment Guideline.**

Table 1-3

Equipment		
Brand	**Description**	**Catalog Number**
-	Cryostat	-
-	Benchtop centrifuge	-
-	Pipettes	-
-	pH meter	-
	Metal heating block dry bath (optional)	-
-	Vortex mixer	-
-	Slide Dryer	-
-	Incubator	-
-	Magnetic Separation Rack for 15 mL tubes	-
-	Slide Dryer	-
Bio-Rad*	T100 Thermocycler	1861096
ABI*	ProFlex 3 x 32-well PCR System	4483636
NEBNext®	Magnetic Separation Rack for <200 µL tubes	S1515S

Equipment		
Brand	**Description**	**Catalog Number**
Thermo Fisher Scientific	Magnetic rack DynaMag™-2 for 1.5-2mL tubes	12321D
	Qubit™ 3 fluorometer	Q33216
Agilent Technologies™	Agilent 2100 bioanalyzer	G2939AA (or similar)

Choose either one of the listed brands (with * mark).

Reagents		
Brand	**Description**	**Catalog Number**
-	100% Ethanol (Analytical grade)	-
Ambion	Nuclease-free water	AM9937
	20X SSC	AM9770
	1X TE buffer, pH 8.0	AM9858
*Agencourt	AMPure® XP	A63882
*Beckman Coulter	SPRIselect	B23317/B23318/ B23319
*VAZYME	VAHTSTM DNA Clean Beads	N411-02
Sigma Aldrich	Hydrochloric acid, HCl	2104-50ML
	Methanol	34860-1L-R
SAKURA	SAKURA Tissue-Tek® O.C.T. compound	4583
Invitrogen	Qubit ssDNA Assay Kit	Q10212
	Qubit dsDNA HS Assay Kit	Q32854
Agilent Technologies™	High sensitivity DNA kit	5067-4626
	High sensitivity RNA kit	5067-1513

Choose either one of the listed brands (with * mark).

Consumables		
Brand	**Description**	**Catalog Number**
-	Stainless-steel base mold	-
-	Aluminum foil	-
-	Forceps	-
-	Microscope glass coverslip (area: 24 mm x 24 mm, thickness: 0.13 - 0.16 mm)	-
-	Microscope glass coverslip (area: 24 mm x 32 mm, thickness: 0.13 - 0.16 mm)	-
Thermo Fisher Scientific	Nunc™ EasYDish™ 60 mm Culture Dish	150462
Corning	Corning® 100 mm TC-treated Culture Dish	353003
	Costar® 6-well Clear TC-treated Well Plates	3516
	50 mL centrifuge tubes	430829
	15 mL centrifuge tubes	430791
Kimtech	Kimwipes™ delicate task wipes	34155
MATIN	Power dust remover	M-6318
Axygen	1.5 mL centrifuge tubes	MCT-150-A
	0.2 mL PCR tubes*	PCR-02-C
	96-well PCR plate*	PCR-96M2-HS-C
	1,000 µL filter tips	TF-1000-L-R-S
	200 µL filter tips	TF-200-L-R-S
	100 µL filter tips	TF-100-R-S
	10 µL filter tips	TXLF-10-L-R-S
	0.5 mL thin wall PCR tubes	
Invitrogen	Qubit Assay Tubes	Q32856
BIOSHARP	Metal Block	-

Choose either one of the listed brands (with * mark)

1.5. Precautions and Warning

- This product is intended for research use only, not for use in diagnostic procedures. Please read all instructions in this manual carefully before using the product.
- Before performing experiments with the kits, users are recommended to ensure that they are familiar with related instruments, and operate them according to manufacturer's instructions..
- Instructions provided in this manual are intended for general use only and optimization may be required for specific applications.
- Thaw reagents in the kits properly prior to use. For enzymes, centrifuge briefly and keep them on ice until further use. For other reagents, thaw them first at room temperature followed by inverting several times to mix them properly, and centrifuge them briefly before placing on ice for further use.
- mRNA capture will be compromised or absent for any scratched areas on the front-side surface of the chip.
- To prevent cross-contamination, we recommend the use of filtered pipette tips. Use a new tip each time for pipetting different solutions.
- Improper handling of samples and reagents may contribute to aerosol contamination of PCR products, resulting in data inaccuracy. Therefore, we recommend two distinctly separated working areas in the laboratory for PCR reaction preparation and PCR product cleanup tests. Use designated pipettes and equipments for each area and execute regular cleaning (with 0.5% sodium hydrochloride or 10% bleach) to ensure a clean and sterile working environment.
- Do not consume any sample or reagent, and avoid direct contact of reagents with skin and eyes. In case of an accident, immediately wash the affected area thoroughly with a large amount of water. Seek emergency medical assistance if needed.

CHAPTER 2
SAMPLE PREPARATION

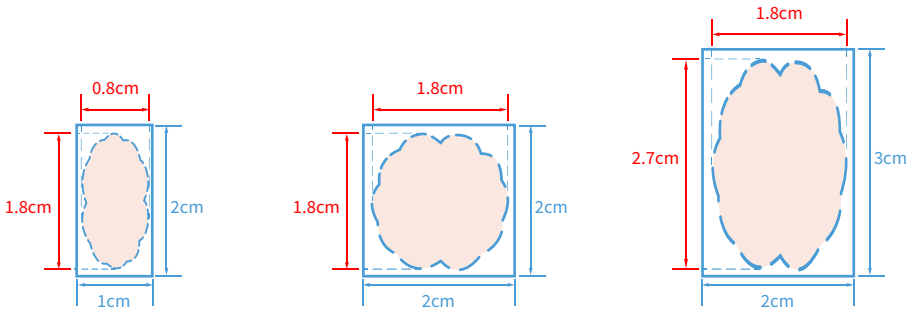
2.1. Sample Requirements for Fresh Frozen Tissue

To avoid RNA degradation, we recommend performing tissue embedding **within 30 min** upon harvesting.

The tissue size should not exceed:

- **0.8 cm x 1.8 cm x 1 cm (for 1cm*2cm Stereo-seq Chip);**
- **1.8 cm x 1.8 cm x 1 cm (for 2cm*2cm Stereo-seq Chip);**
- **1.8 cm x 2.7 cm x 1 cm (for 2cm*3cm Stereo-seq Chip);**

as the tissue section should not exceed 80% area coverage of the chip.



Typically, ~10 tissue slices will be consumed at each step (RIN quality check, Stereo-seq permeabilization optimization, and Stereo-seq Transcriptomics workflow). Please check beforehand whether the embedded tissue thickness can satisfy the need of the experimental design.

Sample Types

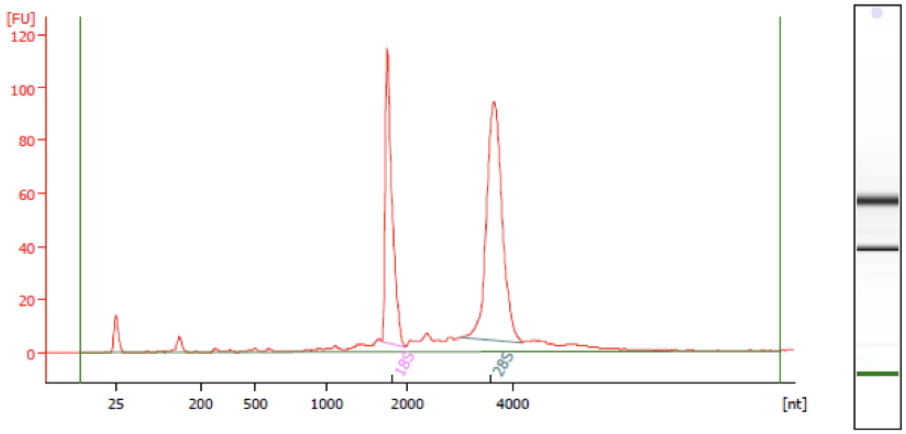
This set of kits can be used for samples from all common animals, including but not limited to human, monkey, and mouse.

For details, please refer to the list: **https://en.stomics.tech/resource/STOmicsTestedTissueList?lang=en#**

Fresh Frozen Sample RNA Integrity Number (RIN) Value

It is recommended to check the RNA quality (RIN value) of a tissue sample before proceeding to Stereo-seq experiment. Total RNA can be extracted from 10-20 slices of 10 μm-thick tissue sections and stored at -20°C in a pre-cooled 1.5mL EP tube. Please refer to Figure 1 for a qualified sample RIN pattern in mouse brain tissue sections.

It is strongly recommended to proceed only with tissue samples with a RIN value ≥7.



Overall Results for sample 6 : 8522203007050

RNA Area:	568.4	RNA Integrity Number (RIN):	9.8 (B.02.11, Anomaly Threshold(s) manually adapted)
RNA Concentration:	281 ng/µl		
rRNA Ratio [28s / 18s]:	1.6		
		Result Flagging Color:	
		Result Flagging Label:	RIN: 9.80

Fragment table for sample 6 : 8522203007050

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,675	1,964	151.3	26.6
28S	3,006	4,187	238.4	41.9

Figure 1. Example of RNA size distribution and RIN value measurement of mouse brain tissue sections.

2.2. Sample Embedding

For a demonstration video of tissue embedding, please refer to the link or by scanning the QR code:
https://drive.google.com/drive/folders/1O138SbfP8lKkYLaScnPkU3pOvwMf0NTW?usp=sharing

a. Prepare these apparatuses/materials in advance:

Materials		
Brand	**Description**	**Quantity**
-	Crushed ice in a box	1
-	Dry ice in a box	1
-	Aluminum foil	1
-	Sealable plastic bag	1
BIOSHARP/Metal Coolbox/ BC032	Metal Block	1
-	Sterile gauze	2
Corning	Corning® 35 mm TC-treated Culture Dish (353001)	1
Sakura/Base Molds/4583	O.C.T	1
Sakura Base Molds 4131/4132/4133 or other brand that's suitable for the tissue size.	Stainless-steel base mold A	1
Sakura Base Molds 4133/4165/4124 or other brand that's suitable.	Stainless-steel base mold B	1
-	Blunt end forceps	1

Materials		
-	Syringe	1
-	Spatula	1
-	Scissors	1
	Stainless Steel Ruler	1

a1. A box of crushed ice and pre-cool OCT on ice for **10 min** in advance.

a2. **2** pieces of stainless-steel base molds slightly larger than the tissue of your interest - mold A and mold B (slightly larger than mold A).

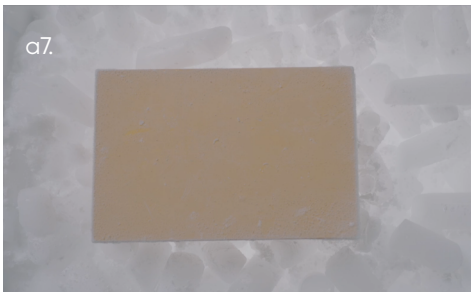
a3. Add a few drops of pre-cooled OCT in the mold A until it reaches approximately 2/3 of the mold and pre-cool on ice for **> 10 min** (remove introduced air bubble using a syringe).

a4. A petri dish filled with OCT and pre-cool it on ice for **> 10 min** (remove introduced air bubble using a syringe).

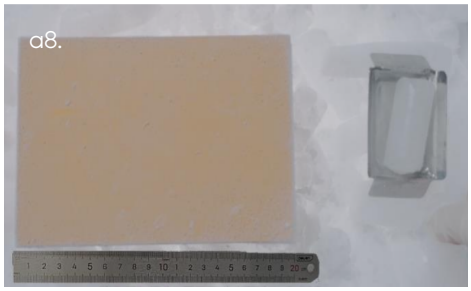
a5. A box of dry ice.

a6. A metal block that has a flat surface to support the stainless-steel base mold when placed on dry ice. The size of the metal block should be larger than the stainless-steel base mold.

a7. Place the metal block on dry ice and pre-cool for **> 5 min** with the flat surface facing up.

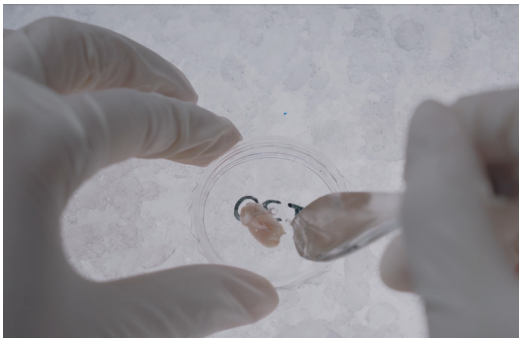


a8. Place mold B and stainless steel ruler on dry ice and pre-cool for **> 5 min**.



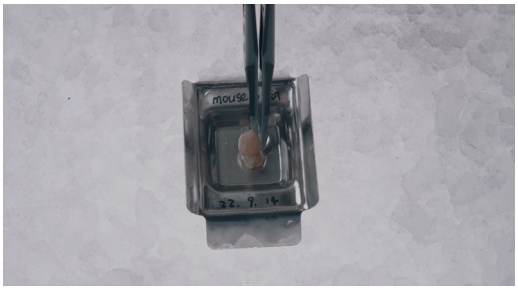
b. Upon harvesting within **30 min**, use sterile gauze or dust-free paper to absorb excess liquid on the tissue surface to avoid ice formation in later steps.

c. Place the tissue in pre-cooled OCT and wrap the tissue evenly with OCT using a spatula without introducing air bubbles.

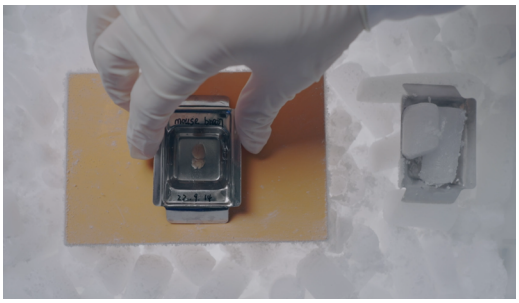


d. Remove any air bubbles using a syringe.

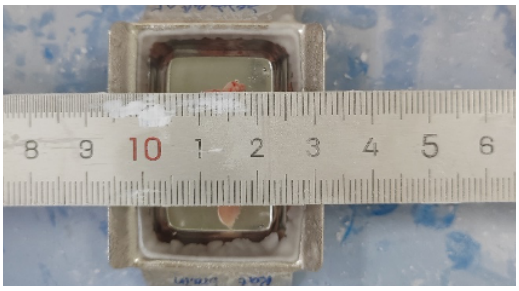
e. Orient the tissue to have the side intended to be sectioned facing downwards and then place into mold A. Make sure the tissue is at the bottom of mold A and fill the mold with cooled OCT without introducing bubbles until the tissue is fully covered by OCT.

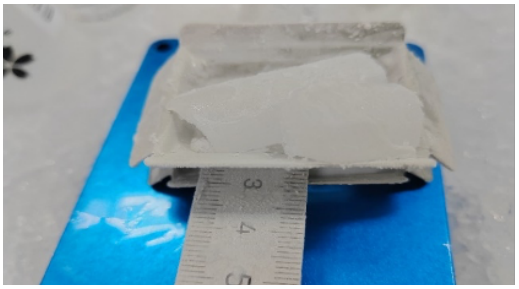


f. Place the tissue-containing mold A onto the metal block that was placed on dry ice.



g. Place the pre-cooled stainless steel ruler on top of mold A (place it on the long side of the container to prevent compression of the tissue). Use mold B as a lid with opening facing up, place on top of mold A gently and then place a few dry ice cubes on top of mold B. Make sure the two stainless steel base molds can be covered with enough dry ice cubes.





h. After **5 min**, remove mold B and the stainless steel ruler then check if the OCT is completely frozen and turns opaque, otherwise repeat g.

i. If the tissue block has solidified and turned opaque, grip the two edges of mold A and press down the edges to detach the tissue block from the mold.

j. Check if the sectioning side of the tissue has been completely covered by OCT. If not, place the tissue block on the metal block, sectioning side facing up, add a few drops of the OCT and then wait till it solidifies and turns opaque.

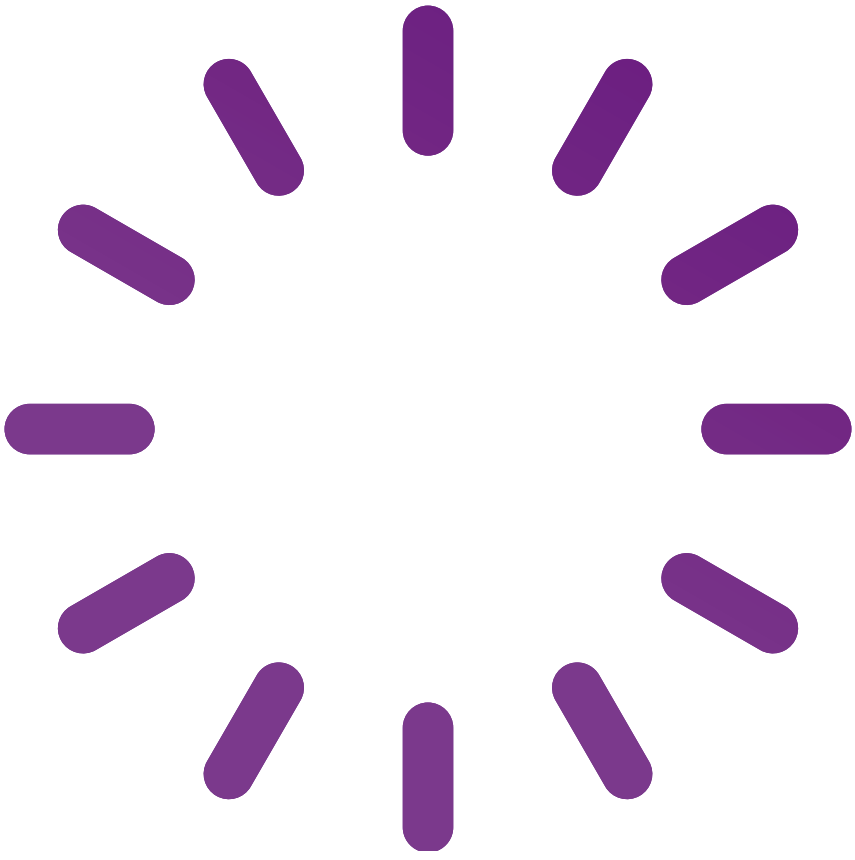
k. Label the tissue block to mark the orientation of the tissue.

2.3. Sample Storage and Transportation

For storing, wrap the tissue block with aluminum foil and keep it in a properly labeled sealable plastic bag to prevent dehydration and damage then store at -80°C. For transportation, please ship samples on dry ice according to local policy.

CHAPTER 3

Stereo-seq TRANSCRIPTOMICS SET FOR LARGE CHIP STANDARD OPERATING PROCEDURE



3.1. Experimental Preparation

Unless otherwise specified, nuclease-free water is used for all reagents being prepared prior to this experiment.

Prep Day	Reagent	Preparation Steps for 1cm * 2cm Chip T	Preparation Steps for 2cm * 2cm Chip T	Preparation Steps for 2cm * 3cm Chip T	Maintenance
Day 1	5X SSC	Dilute 5 mL of 20x SSC to 20 mL			Room Temperature
	0.1X SSC	Dilute 100 µL of 20x SSC to 20 mL; Dilute 250 µL of 20x SSC to 50 mL			Room Temperature
	Wash Buffer	Prepare at least **150** µL per chip (**142.5** µL 0.1X SSC with **7.5** µL RI).	Prepare at least **300** µL per chip (**285** µL 0.1X SSC with **15** µL RI).	Prepare at least **400** µL per chip (**380** µL 0.1X SSC with **20** µL RI).	On ice until use
	0.01N HCl	Prepare at least 2mL of 0.01N HCL per sample. Configure HCL to 0.01N. Measure and make sure the pH = 2 (Prepare at least 2 mL / sample).			Room temperature for 48 hr **(Storing longer than 48 hr will affect the desired pH. Please use WITHIN 48 hr of preparation)**
	ALWAYS use freshly prepared 0.01N HCl (pH = 2.0 ± 0.1). For pre-made 0.1N HCl and newly purchased HCl, check the pH prior to the experiments.				
	10X Permeabilization Reagent Stock Solution	Add 1 mL of freshly prepared 0.01N HCl to dissolve PR Enzyme (red cap, in powder form), and thoroughly mix the reagent through pipetting.			-20°C
	DO NOT vortex the permeabilization enzyme. Mix by pipette before using. **Aliquot this 10X stock solution to avoid freeze-thaw cycles.**				
	1X Permeabilization Reagent Solution	Make 1X PR solution (at least **150 µL** / chip) by diluting 10X PR stock solution with 0.01N HCl.	Make 1X PR solution (at least **300 µL** / chip) by diluting 10X PR stock solution with 0.01N HCl.	Make 1X PR solution (at least **650 µL** / chip) by diluting 10X PR stock solution with 0.01N HCl.	On ice until use, up to 6 hr

Prep Day	Reagent	Preparation Steps for 1cm * 2cm Chip P	Preparation Steps for 2cm * 2cm Chip P	Preparation Steps for 2cm * 3cm Chip P	Maintenance
Day 1	RT Oligo	Short spin the primer tube, dissolve RT Oligo in 79 μL TE buffer. Close the lid, vortex the tube for 15 sec at highest speed and short spin the tube.			-20°C
	Aliquot the unused RT Oligo to avoid freeze-thaw cycles and store at -80°C.				
	Glycerol	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.			Room Temperature
	PR Rinse Buffer	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.			Room Temperature
	PR Rinse Buffer (with 5% RI)	Prepare at least **150** μL per chip (**142.5** μL 0.1X SSC with **7.5** μL RI).	Prepare at least **300** μL per chip (**285** μL 0.1X SSC with **15** μL RI).	Prepare at least **400** μL per chip (**380** μL 0.1X SSC with **20** μL RI).	On ice until use
Day 2	80% Ethanol	Dilute 100% ethanol to 80%			Room Temperature up to 1 day
	Magnetic beads	Take it out in advance and equilibrate to room temperature at least 30 min prior to use.			4°C

Other Preparation	Consumables Required		
Steps	**1cm * 2cm Chip T**	**2cm * 2cm Chip T**	**2cm * 3cm Chip T**
Chip Placement	100-mm Culture Dish (Place parafilm at the bottom of the culture dish)		
Prepare a parafilm piece in a suitable size and attach it to the petri dish bottom. Gently press and fix the edges of the parafilm with the back-end of the forceps. Place your chips on the parafilm throughout experiments to prevent chips from sliding and colliding into the wall of the petri dish.			
Chip Washing	6-well plate		60-mm Culture Dish
Methanol Fixation			
Glycerol Removal			
Tissue Removal			
cDNA Release			

Other Preparation		
Equipments	**Set up**	**Note**
Cryostat	Set the cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C~-15°C.	The specimen disc temperature depends on the tissue type.
Turn on the incubator	Set the temperature in the following order: 37°C for chip drying and permeabilization; 42°C for reverse transcription; 55°C for tissue removal.	Check if there is any abnormality with the incubator and replace it if necessary.
Fluorescence Microscope	Set the epi-fluorescence channel to FITC mode.	Room Temperature

3.2. Cryosection Preparation

a. Set the slide dryer to 37°C or the PCR thermal cycler to 37°C in advance with a PCR adaptor.

b. Set cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C~-15°C.

If the specimen disc is over-cooled, it could lead to tissue section cracking during sectioning, while sections would wrinkle when the disc temperature is too high. Optimal specimen disc temperature depends on the tissue type.

c. Place forceps, brushes, and razor blades inside the chamber for pre-cooling.

d. Take the OCT-embedded tissue sample out of the -80°C freezer to the chamber and allow it to equilibrate to the cryostat chamber temperature (Equilibration time should vary depending on the size of the tissue sample. In order to ensure a smooth tissue sectioning process, tissue sizes of 2 cm x 3 cm x 1 cm should equilibrate for about **1 hr** as a reference).

e. Remove the sample outer covers (aluminum foil) and trim the embedded tissue block into appropriate size (sectioning area should be smaller than 0.9 cm x 1.8 cm for 1cm*2cm Stereo-seq Chip, 1.8 cm x 1.8 cm for 2cm*2cm Stereo-seq Chip, and 1.8 cm x 2.7 cm for 2cm*3cm Stereo-seq Chip).

f. Use OCT to mount the embedded tissue block onto the specimen disc/holder of the cryostat chamber.

g. Do a final trimming if necessary to ensure a good fit between the tissue section and Stereo-seq Chip later. Now, the specimen is ready for cryosection.

3.3. Tissue Mounting

a. Take the Chip T out of the vacuum sealed aluminum bag and record Chip ID (SN) number that is on the back side of the chip. Make sure not to touch the front side chip surface.

The front-side of a chip has a shiny surface which contains DNB-probes for mRNA capture. DO NOT scratch the surface.

b. Equilibrate Stereo-seq Chip T to room temperature for **1 min** within the parafilm attached 100-mm Culture Dish.

c. Rinse Stereo-seq Chip T:

1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
6-well plate	6-well plate	60-mm Culture Dish
Rinse the chip with **3000 µL** nuclease-free water **twice** with a pipette		Rinse the chip with **4000 µL** nuclease-free water **twice** with a pipette

d. Remove excess water on the chip by blowing gently with a power dust remover (MATIN, M-6318) from one side of the chip at a 30~45-degree angle horizontal to the plane of the chip. Wipe excess water around the chip and on the slide with dust-free paper.

e. Only when the chip is completely dry and without wavy white stains is it ready for tissue mounting.

f. Pre-cool methanol:

1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
6-well plate	6-well plate	60-mm Culture Dish
Add **2-4 mL** of methanol into one well of the 6-well plate for one chip and pre-cool for **5-30 min** at -20°C		Add **3-5 mL** of methanol into the petri dish for one chip and pre-cool for **5-30 min** at -20°C

g. Tissue mounting could be achieved via either **cold method** (option A) or **warm method** (option B).

A. Cold Method

1) Place Stereo-seq Chip inside the chamber with the front-side facing up and pre-cool inside the cryostat chamber for **3~10 min.**

Prolonged cooling for over 10 min may cause mist formation on the chip surface.

2) Perform cryosection, then carefully flatten the tissue section out by gently touching the surrounding OCT with cryostat brushes. Place a tissue section onto the chip center carefully with forceps and brushes. Make sure the tissue section is complete and without wrinkles.

3) Immediately pick up the Stereo-seq Chip and place a finger on the backside of the chip for a few seconds to warm up and allow the section to adhere to the chip.

It is recommended to finish tissue section placement within 5 min.

4) Once complete all tissue mounting, immediately dry the Stereo-seq Chip at 37°C for:

Table 3-1 Drying time for Stereo-seq Large Chip Designs

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
Chip drying time:	8 min	8 min	10 min

B. Warm Method

1) Perform cryosection, carefully flatten the tissue section out by gently touching the surrounding OCT with cryostat brushes, and move the tissue section to the edge.

2) Hold the chip at one corner with a pair of forceps, turn it around to have its active surface facing downward, and match it onto the tissue section.

3) Place it on top of the tissue section and let the tissue adhere on to the chip.

4) Turn the chip again to make the tissue-mouted front-side facing upward, and immediately dry the Stereo-seq Chip at 37°C according to the time listed in Table 3-1.

3.4. Tissue Fixation

a. After drying, immediately immerse the tissue-mounted chip in pre-cooled methanol prepared in section 3.3-f for a **40-min** fixation at -20°C.

b. After fixation, move the 6-well plates or 60 mm culture dish to a sterile fume hood.

c. Take out the chip and absorb excess methanol on the chip from its corner and back with a Kimwipes™ Delicate Task Wipes.

d. Place the chip in the 100mm petri dish with a parafilm attached to the bottom and do not close the lid.

e. Leave the petri dish in the fume hood for **4-6 min** to let the methanol fully evaporate.

f. While waiting, prepare fluorescent staining solution according to Table 3-2 and store it in the dark. **【PREPARE AHEAD】**

Table 3-2 Tissue fluorescent staining solution

Components	1X (μL) for 1cm * 2cm Chip T	1X (μL) for 2cm * 2cm Chip T	1X (μL) for 2cm * 3cm Chip T
5X SSC	189	378	756
Qubit ssDNA Reagent	1	2	4
RI	10	20	40
Total	200	400	800

g. Once methanol is fully evaporated, transfer the Stereo-seq Chip onto a flat and clean bench top surface.

3.5. Fluorescent Staining

a. Place the Stereo-seq Chip in a clean 10cm petri dish, add tissue fluorescent staining solution according to Table 3-2 by first pipetting one droplet at each corner of the chip and then adding the rest of the staining solution to the middle to merge all the droplets.

Ensure that the chip is completely covered by tissue fluorescent staining solution.

b. Cover the petri dish with aluminum foil and incubate it for **5 min** at room temperature in the dark.

c. Slightly tilt the Stereo-seq Chip T while gently removing the staining solution from the corner of the chip using a pipette. Try to remove as much solution as possible.

d. Add Wash Buffer and then remove the solution to wash the chip:

Table 3-3 Wash Buffer addition volume for Stereo-seq Large Chip Designs

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
Wash Buffer addition volume	**150 μL / chip**	**300 μL / chip**	**400 μL / chip**

e. Slightly tilt the Stereo-seq Chip T, remove Wash Buffer from the corner of the chip using a pipette.

f. Carefully transfer the chip on to the Kimwipe with a pair of forceps, remove excess water on the chip by blowing gently with a power dust remover (MATIN, M-6318) from one side of the chip at a 30~45-degree angle horizontal to the plane of the chip.

Ensure no residual solution is left on the chip.

g. Pipette glycerol gently onto the center of the chip without introducing air bubbles according to Table 3-4:

Table 3-4 Glycerol addition volume for Stereo-seq Large Chip Designs

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
Glycerol addition volume	**8-12 μL / chip**	**16-24 μL / chip**	**24-36 μL / chip**

h. With a pair of forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chip. Ensure that the chip is completely covered by glycerol and the coverslip. To avoid fluorescent bleaching, **IMMEDIATELY proceed to imaging**.

Make sure the coverslip is clean without any dust or debris. Use a alcohol swab or blow with a power dust remover to clean the cover slips if required.

3.6. Imaging

a. Create a new folder in a fluorescent microscope-connected PC, name it with the chip ID number and other essential information.

Only use letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.
Example: B00249A1B2

b. Place 1-2 μL of water on the imaging platform first, then transfer and place the chip onto the water drop. Water surface tension will grab onto the chip and adhere it onto the imaging platform.

c. Remove the light shield and select the chip area of interest.

d. Take fluorescence images from the chip with the following microscope setting: FITC channel, 10X objective lens, full scan on capture area.

Take fluorescent images immediately to avoid prolonged fluorescent exposure which causes bleaching.

e. Save original tile (FOV) images files and stitched images.

Glycerol mounted chips could be stored for a maximum of 4 hr after imaging.

f. Open the ImageStudio software and the Image Quality Control functional module within the software. Upload your nuclei-stained (ssDNA) image and run Image QC according to the ImageStudio User Manual within the software.

The captured ssDNA-stained image needs to pass ImageQC in order to proceed to further image analysis (image "register") in Stereo-seq Analysis Workflow (SAW) pipelines.

If Image QC failed, continue with the experimental procedures and later perform optimal image analysis under the guidance of your local Field Application Scientist.

g. After imaging, gently push the coverslip with a pair of forceps until it is slightly beyond the edge of Stereo-seq Chips.

h. Grip onto the coverslip with the pair of forceps and pull it to slide over the Stereo-seq Chips slowly until the chips and the coverslip are fully separated.

i. Place Chip T (1cm * 2cm) / Chip T (2cm * 2cm) in a 6-well plate or Chip T (2cm * 3cm) in a 60mm culture dish then wash with 0.1X SSC according to Table 3-5.

Table 3-5 Glycerol removal and washing

1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
6-well plate	6-well plate	60-mm Culture Dish
Gently rinse the chip with **1500 µL** 0.1X SSC with a pipette around each corner of the chip in the well up and down for about 5 times gently. Release the solution drop by drop directly onto the chip surface twice, and then pipette the solution up and down next to the chip three times.		Rinse the chip with **3000 µL** 0.1X SSC with a pipette around each corner of the chip in the culture dish up and down for about 5 times gently. Release the solution drop by drop directly onto the chip surface twice, and then pipette the solution up and down next to the chip three times.

Ensure the chips are covered with 0.1X SSC solution completely.

j. Discard 0.1X SSC solution then repeat step i.

k. Transfer the chip onto dust-free paper and absorb excess solution from the edge and the back of the chip.

l. Place the chip into a 100mm-petri dish with parafilm attached to the bottom and immediately proceed to tissue permeabilization.

3.7. Tissue Permeabilization

a. Set aside the 0.01N HCl and 1X Permeabilization Reagent Solution you prepared in **3.1 Experimental Preparation**.

b. Make sure your incubator has been switched on and set to 37°C.

c. Prewarm the 1X PR solution in the 37°C incubator for **10 min**.

d. Thaw RT QC Reagent, RT Additive and RT QC Enzyme on ice.

e. Place the chip-containing petri dish in the incubator then add 1X permeabilization Reagent Solution onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets according to Table 3-6.

Table 3-6 1X PR solution addition volume for Stereo-seq Large Chip Designs

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
1X PR solution addition volume	**150 μL / chip**	**300 μL / chip**	**650 μL / chip**

Make sure the chip is completely covered with 1X Permeabilization Reagent Solution.

f. Incubate at 37°C for the optimal permeabilization time.

Optimal permeabilization time for each size is pre-determined by the Stereo-seq Permeabilization Kit, 4RXN (111KP004). For more information, refer to the user manual of Stereo-seq Permeabilization Set for Stereo-seq Large-sized Chip (≤ 2cm * 3cm) User Manual.

g. While waiting for permeabilization to be done, prepare RT mix according to Table 3-7 and leave it on ice until use. **【PREPARE AHEAD】**

Table 3-7 RT Mix

Components	1X (μL) for 1cm * 2cm Chip T	1X (μL) for 2cm * 2cm Chip T	1X (μL) for 2cm * 3cm Chip T
RT Reagent	136	256	336
RT Additive	8.5	16	21
RI	8.5	16	21
RT Oligo	8.5	16	21
ReverseT Enzyme	8.5	16	21
Total	170	320	420
Volume Addition per chip	150	300	400

h. Take the chip-containing petri dish out of the incubator.

i. Slightly tilt the petri dish then remove 1X Permeabilization Reagent Solution with a pipette from a corner of the chip. Make sure to remove as much solution as possible.

j. Add PR Rinse Buffer (with 5% RI) on to the chip according to Table 3-8.

Table 3-8 PR Rinse Buffer (with 5% RI) addition volume for Stereo-seq Large Chip Designs

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
PR Rinse Buffer (with 5% RI)	**150 μL / chip**	**300 μL / chip**	**650 μL / chip**

k. Remove PR Rinse Buffer (with 5% RI) from a corner of the chip using a pipette, keep the chip moisturized.

Make sure to not dry the chip completely.

l. Transfer the chip to a new petri dish with parafilm attached to the bottom and then continue with reverse transcription immediately to avoid RNA degradation.

3.8. Reverse Transcription

a. Make sure the temperature of the incubator has been set to 42°C in advance.

b. Gently add RT QC Mix on to the chip from the corners according to Table 3-7. Make sure the chip is completely covered with RT Mix.

c. Wrap the chip-containing petri dish with parafilm and incubate it at 42°C for **5 hr** or longer (no more than 16 hr) for the reverse transcription reaction to take place.

If incubation lasts overnight, use parafilm to tightly seal the petri dish which contains the chip, then wrap the petri dish with plastic wrap to prevent evaporation.

3.9. Tissue Removal

Prepare		
Reagent	**Preparation Steps**	**Storage**
TR buffer	Heat the buffer for **5 min** at 55 °C to dissolve the precipitate. Equilibrate it to room temperature prior to use.	Room Temperature
cDNA Release Buffer	Heat the buffer for **5 min** at 55 °C to dissolve the precipitate. Equilibrate it to room temperature prior to use.	Room Temperature

If white precipitate is observed in the buffer, dissolve them by heating the buffer at 55 °C again and equilibrate to room temperature before mixing.

a. Check and make sure the incubator temperature is set to 55°C.

b. Take the chip-containing petri dish out of the 42°C incubator.

c. Slightly tilt the petri dish, remove RT Mix from the chip surface with a pipette.

d. Add 0.1X SSC solution on to the chip according to Table 3-9.

Table 3-9 0.1X SSC addition volume for Stereo-seq Large Chip Designs post RT

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
0.1X SSC addition volume	**150 μL / chip**	**300 μL / chip**	**400 μL / chip**

e. Slightly tilt the petri dish, remove 0.1X SSC with a pipette from the corner of the chip.

f. Transfer Stereo-seq Chip T into a 6-well plate (1cm*2cm or 2cm * 2cm) or 60mm Culture Dish (2cm*3cm).

g. Add TR Buffer onto the chip according to Table 3-10.

Table 3-10 TR Buffer addition volume for Stereo-seq Large Chip Designs

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
TR Buffer addition volume	**1500 μL / chip**	**2000 μL / chip**	**3000 μL / chip**

h. Incubate the chip at 55°C for **10-30 min**.

i. While waiting, prepare cDNA Release Mix according to Table 3-11 and leave at room temperature until use. **【PREPARE AHEAD】**

Table 3-11 cDNA Release Mix

Components	1X (μL) for 1cm * 2cm Chip T	1X (μL) for 2cm * 2cm Chip T	1X (μL) for 2cm * 3cm Chip T
Reaction reservoir		6-well plate	60-mm Culture Dish
cDNA Release Buffer		55	155
cDNA Release Enzyme		1045	2945
Total		1100	3100
Volumn Addition per chip		1000	3000

j. At the end of incubation, remove the Stereo-seq Chip T from the incubator.

k. Slightly tilt the 6-well plate or 60-mm culture dish, remove TR Buffer with a pipette from the chip.

If tissue removal is not complete, add 0.1X SSC according to Table 3-12 and pipette up and down the well to remove the remaining tissue on the chip. Slightly tilt the 6-well plate or 60-mm culture dish, remove 0.1X SSC solution from the corner of the well without touching the chip surface.

Table 3-12 0.1X SSC addition volume for Stereo-seq Large Chip Designs post TR

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
0.1X SSC addition volume	**1500 µL / chip**	**2000 µL / chip**	**3000 µL / chip**

3.10. cDNA Release and Collection

a. Add cDNA Release Mix prepared in 3.9-i.

b. Use parafilm to tightly seal the well or petri dish which contains the chip then cover the lid and further seal the plate or petri dish with plastic wrap to prevent evaporation. Incubate at 55°C for **3 hr** or longer (no longer than 18 hr).

DNA collection step may be left overnight. If it is left overnight, make sure the well plate or petri dish has been sealed tightly with the parafilm.

c. After the reaction, completely collect the cDNA Release Mix:

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
cDNA collection	Completely collect the cDNA Release Mix from each well into a new **5 mL** tube for 1.5-2mL magnetic separation rack; or into a new **15mL** tube for 15mL magnetic separation rack		Completely collect the cDNA Release Mix from each well into a new **15mL** tube for 15mL magnetic separation rack

d. Add nuclease-free water in **two additions**. Pipette up and down to wash the chip surface thoroughly and then collect it into the same **5 mL** or **15mL** tube with the cDNA Release Mix.

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
Nuclease-free water addition volume	1st addition: **500 µL / chip** 2nd addition: **500 µL / chip**		1st addition: **1500 µL / chip** 2nd addition: **1500 µL / chip**
Total	**1000 µL / chip**		**3000 µL / chip**

Make sure to collect as complete as possible to retrieve enough cDNA on the chip. cDNA Release Mix should be about 1000 µL/3000 µL after incubation (the volume might be less than 1000 µL/3000 µL). It is required to combine the collected cDNA Release Mix with the 1000 µL/3000 µL nuclease-free water before proceeding to the next step.

3.11. cDNA Purification and Amplification

Background Information

For bead-based purification, we recommend using DNA Cleanup Beads AMPure® XP(Agencourt, Cat. No. A63882), SPRIselect (Beckman Coulter, B23317/B23318/B23319) or VAHTSTM DNA Clean Beads (VAZYME, Cat. No. N411-02). *If magnetic beads from other sources are used, please optimize the cleanup conditions before getting started.*

Before Use

- To ensure the DNA capture efficiency of the magnetic beads, equilibrate the beads to room temperature **30 min before use**.
- Vortex or pipette up and down to ensure the beads are thoroughly mixed every time before use.
- The amount of magnetic beads directly affects the distribution of purified DNA fragments.

Operation Notes

- In the magnetic separation step, please allow the solution to become completely clear before removing the supernatant. This process usually takes approximately **2-3 min**, but can be longer or shorter depending on the type of magnetic separation rack being used.
- When collecting the supernatant with a pipette after magnetic separation, avoid taking up the beads. Instead of collecting the entire supernatant fraction, leave 2-3 µL in the tube to avoid the pipette from direct contacting the beads. If the beads are mistakenly taken up, dispense everything and redo the magnetic separation.
- Use freshly prepared 80% ethanol (at room temperature) to wash the beads. Keep the sample tube on the magnetic separation rack during the washing step. Do not shake or disturb the beads.
- After the 2nd wash of beads with ethanol, try to remove all the liquid within the tube. You may centrifuge briefly to accumulate any remaining liquid at the bottom of the tube, then separate beads magnetically, and remove the remaining liquid by using a small volume pipette.
- After washing twice with ethanol, air-dry the beads at room temperature. Drying usually takes approximately **5-10 min** depending on the lab temperature and humidity. Watch closely until the pellet appears sufficiently dry with a matte appearance, then continue to the elution step with TE Buffer.
- During the elution step, do not touch the beads with the pipette tip when removing the supernatant. Contamination of a DNA sample with beads may affect subsequent purification steps. Therefore, to avoid the pipette tip from direct contacting the beads, always collect the eluate in 2 µL less than the initial volume of TE buffer used for the elution.
- **Pay attention when opening/closing the lid of a sample tube on a separation rack. Strong vibrations may cause samples or beads to spill from the tubes. Hold the body of the tube while opening the lid.**

a. Equilibrate the magnetic beads to room temperature for at least **30 min**.

b. If white precipitate is observed in the collected cDNA, dissolve it by heating at 55°C and equilibrate to room temperature before the puritification step.

c. cDNA Purification Procedures with 0.8X Magnetic Bead

【1.5-2mL Magnetic Separation Rack】

1) Aliquot the collected cDNA:

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
Aliquot in 1.5mL eppendorf tubes	Aliquot into four 1.5mL tube		Aliquot into eight 1.5mL tube

Mix the collected cDNA (450-490 μL) with the beads in a ratio of 1 : 0.8. Vortex the mix then incubate it at room temperature for **10 min**.

2) Spin down and place the tube onto a magnetic separation rack for **3 min** until the liquid becomes clear.

3) Carefully remove and discard the supernatant with a pipette (If foams are seen on the cap, discard them with a pipette).

4) Keep the tube on the magnetic separation rack and add 1 mL of freshly prepared 80% ethanol to wash the beads by rotating the tube on the magnetic rack. Incubate for **30 sec** and carefully remove and discard the supernatant.

Always place the pipette tips on the tube wall and away from the magnetic beads. Do not disturb the beads while transferring the supernatant (If foams are seen on the cap, clean the cap with 80% ethanol).

5) Repeat step 4) one more time.

6) Keep the tube on the magnetic rack, and open the lid to air-dry the beads at room temperature until no wetness (reflectiveness) is observed. Drying times will vary but will be approximately **5-8 min**.

7) Add 22 μL of nuclease-free water to the dried beads. Mix the beads and nuclease-free water by vortexing. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic separation rack for **3-5 min** until the liquid becomes clear.

8) Transfer the supernatant (~ **21 μL**) into a new 0.2 mL PCR tube.

9) Add another 22 μL of nuclease-free water to the dried beads in step 7) for a second elution. Mix the beads and nuclease-free water by vortexing. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic separation rack for **3-5 min** until the liquid becomes clear.

10) Transfer the supernatant (~ **21 μL**) into the 0.2 mL PCR tube in step 8) and obtain a combined eluted cDNA (~**42 μL**)

d. If collected eluted cDNA is less than 42 μL, simply top it up with nuclease-free water.

Store the beads with 42 μL of nuclease-free water at 4°C after collecting the eluted cDNA till your cDNA final product has passed QC.

【15mL Magnetic Separation Rack】

1) Aliquot the collected cDNA:

Chip Size:	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T
Aliquot in 1.5mL eppendorf tubes	Aliquot into one 15mL tube		Aliquot into two 15mL tube

Mix the collected cDNA (1000μL/3000μL) with the beads in a ratio of 1 : 0.8. Vortex the mix then incubate it at room temperature for **10 min**.

2) Spin down and place the tube onto a magnetic separation rack for **3 min** until the liquid becomes clear.

3) Carefully remove and discard the supernatant with a pipette (If foams are seen on the cap, discard them with a pipette).

4) Keep the tube on the magnetic separation rack and add 1 mL of freshly prepared 80% ethanol to wash the beads by rotating the tube on the magnetic rack. Incubate for **30 sec** and carefully remove and discard the supernatant.

Always place the pipette tips on the tube wall and away from the magnetic beads. Do not disturb the beads while transferring the supernatant (If foams are seen on the cap, clean the cap with 80% ethanol).

5) Repeat step 4) one more time.

6) Keep the tube on the magnetic rack, and open the lid to air-dry the beads at room temperature until no wetness (reflectiveness) is observed. Drying times will vary but will be approximately **5-8 min**.

7) Add 88 μL of nuclease-free water to the dried beads. Mix the beads and nuclease-free water by vortexing. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic separation rack for **3-5 min** until the liquid becomes clear.

8) Transfer the supernatant (~ **84 μL**) into four new 0.2 mL PCR tube (~21μL per tube).

9) Add another 88 μL of nuclease-free water to the dried beads in step 7) for a second elution. Mix the beads and nuclease-free water by vortexing. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic separation rack for **3-5 min** until the liquid becomes clear.

10) Transfer the supernatant (~ **84 μL**) into the four 0.2 mL PCR tube in step 8) and obtain a combined eluted cDNA (~**42 μL**) in each 0.2mL PCR tube.

d. If collected eluted cDNA is less than 42 μL, simply top it up with nuclease-free water.

Store the beads with 160 μL of nuclease-free water at 4°C after collecting the eluted cDNA till your cDNA final product has passed QC.

e. Prepare PCR Mix by referring to Table 3-13. The total volume for the PCR reaction is 100 μL.

Table 3-13 PCR Mix

Components	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
cDNA Amplification Mix	50	110	165	220
cDNA Primer	8	17.6	26.4	35.2
Eluted cDNA	42	2 x 42	3 x 42	4 x 42
Total	100	2 x 100	3 x 100	4 x 100

f. Mix gently and short spin before placing the reaction tube in a thermal cycler. Amplify the eluted cDNA based on the PCR program stated in Table 3-14.

Table 3-14 PCR Program for Amplification (for 100 μL)

Temperature	Time	Cycle
(Heated lid) 105°C	on	-
95°C	5 min	1
98°C	20 sec	15
58°C	20 sec	
72°C	3 min	
72°C	5 min	1
12°C	Hold	-

g. Prepare Qubit dsDNA Mix and record the concentration of PCR product according to Table 3-15.

Table 3-15 Qubit dsDNA Mix

Components	1X (μL)
Invitrogen™ Qubit dsDNA HS Buffer	198
Qubit dsDNA HS Reagent 200X	1
PCR Product	1
Total	200

h. Vortex the mix and then take 1 μL of the PCR product and measure its concentration using Qubit dsDNA HS Kit. The DNA concentration is usually more than 5 ng/μL.

QC

QC **For troubleshooting purposes, we recommend leaving about 2 μL of the PCR product in a PCR tube.**

i. Use magnetic beads to purify the PCR product in a volume ratio of 1 : 0.6 (DNA : beads).

1) Mix the cDNA PCR product (400 μL for 1cm*2cm and 2cm*2cm Stereo-seq Chip; 800 μL for 2cm*3cm Stereo-seq Chip) with beads in a ratio of 1 : 0.6 in a 1.5mL tube. Vortex the mixture then incubates it at room temperature for **10 min**.

2) Spin down and place the sample tube onto a magnetic separation rack for **3 min** until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.

3) Keep the tube on the magnetic separation rack and add 200 μL of freshly prepared 80% ethanol to wash the beads by rotating the tube on the magnetic separation rack. Incubate for **30 sec** then carefully remove and discard the supernatant.

Always place the pipette tips on the tube wall and away from the magnetic beads. Do not disturb the beads while transferring the supernatant.

4) Repeat step 3) one more time.

5) Keep the tube on the magnetic rack, and open the lid to air-dry the beads at room temperature until no wetness (reflectiveness) is observed. Drying times will vary but will be approximately **5-8 min**.

6) Vortex the dried beads with 42 μL of TE buffer(40 μL for 1cm*2cm and 2cm*2cm Stereo-seq Chip; 80 μL for 2cm*3cm Stereo-seq Chip). Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic rack for **3-5 min** until the liquid becomes clear. Transfer 40 μL supernatant to a new 1.5 mL centrifuge tube.

Stop Point:

- **The purified cDNA sample can be stored at −20°C for up to 1 month.**

For troubleshooting purposes, we recommend storing the beads with 40 μL or 80 μL of nuclease-free water at 4°C after purification till your cDNA final product has passed QC.

j. Take 1 μL of the cDNA sample and measure and record the concentration of the purified cDNA with Qubit dsDNA HS Kit.

k. Analyze the sample (dilution might be required) on an Agilent Bioanalyzer High Sensitivity chip or other library quality control platform such as Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer) and Fragment Analyzer™ (Advanced Analytical).

A qualified cDNA sample should have a main fragment distribution peak appearing at around 1,000- 1,500 bp (Figure 2), and a yield higher than 20 ng.

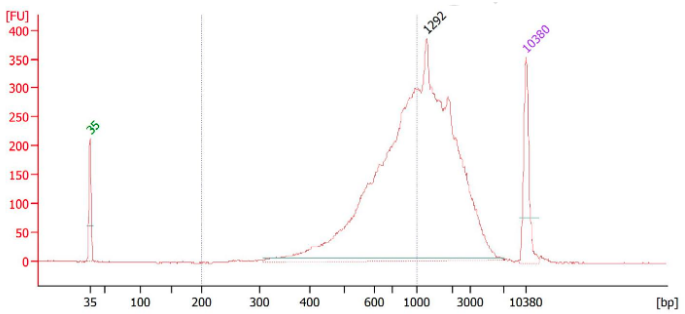
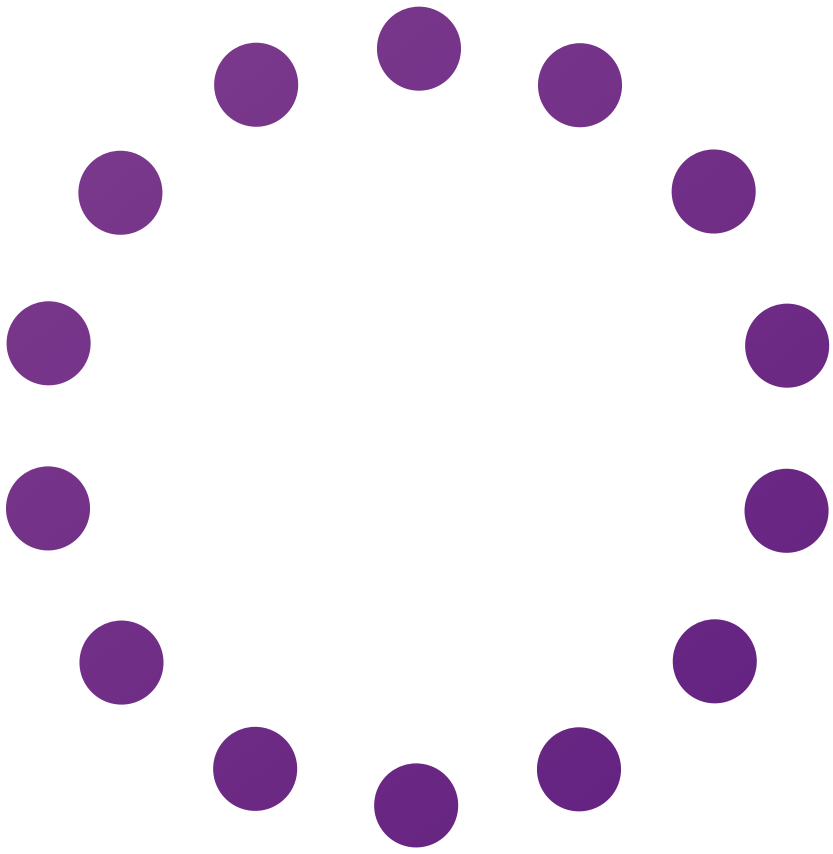


Figure 2. A representative Agilent Bioanalyzer 2100 analysis result of a PCR amplified cDNA sample

CHAPTER 4
LIBRARY PREPARATION



The later steps will require the usage of Stereo-seq Library Preparation kit. Please make sure you have purchased them separately and prepare them in advance before constructing your library.

Stereo-seq Library Prep Kit Cat. No.: 111KL114			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
TME	1000028515	○ (white)	4 μL × 1
Stop Buffer	1000028516	○ (white)	40 μL × 1
TMB	1000028517	○ (white)	40 μL × 1
PCR Barcode Primer Mix (Barcode 57-64)	1000028519	●	50 μL × 1
PCR Barcode Primer Mix (Barcode 81-88)	1000029088	●	50 μL × 1
PCR Barcode Primer Mix (Barcode 89-96)	1000029089	●	50 μL × 1
PCR Barcode Primer Mix (Barcode 97-104)	1000029180	●	50 μL × 1
PCR Amplification Mix	1000029181	●	400 μL × 1

Storage Temperature: -25°C~-18°C | Transported by cold chain | Expiration Date: refer to label

Performance of products may only be guaranteed before their expiration date. Proper performance is also subject to the products being transported, stored, and used in appropriate conditions.

4.1. Experimental Preparation

Unless otherwise specified, nuclease-free water is used for all reagents being prepared prior to this experiment.

Reagent	Preparation Steps	Maintenance
80% Ethanol	Dilute 100% ethanol to 80%	Room temperature up to 1 day
Magnetic beads	Take it out in advance and equilibrate to room temperature at least 30 min prior to use.	4°C
10-fold diluted TME	Dilute 1 μL of TME to 10 μL with TE buffer	On ice up to 1 hr
DO NOT dilute all the TME at once. Volume provided should be enough for 4 dilutions.		
Stop Buffer	Take it out in advance and equilibrate to room temperature at least 30 min prior to use	Room temperature up to 1 day

4.2. cDNA Fragmentation and Amplification

a. Use 20 ng cDNA sample prepared in section 3.11. for the following fragmentation reaction.

b. Prepare the Fragmentation Reaction Mix on ice through gentle pipetting according to Table 4-1. Pipette 10-fold diluted TME up and down before mixing with the rest. After a short spin, gently mix the solution through pipetting while keeping the tube on ice.

Avoid vortexing TME.

Table 4-1 Fragmentation Reaction Mix

Components	1X (μL)
TMB	4
10-fold diluted TME	1
cDNA Product	X
Nuclease-free water	15-X
Total	20

cDNA Input: X (μL) = 20 ng/Concentration of cDNA (ng/μL)

c. Program a thermocycler according to Table 4-2. When the module starts to heat up, put the reaction tube into the thermocycler.

Please DO NOT leave the reaction tube on ice after taking it out of the thermocycler.

Table 4-2 Fragmentation reaction program

Temperature	Time
(Heated lid) 60°C	on
55°C	10 min
4°C	Hold

d. After the fragmentation reaction program is done, take out the reaction tube and leave it at room temperature. Add 5 μL of Stop Buffer to the fragmentation reaction mix to terminate the fragmentation process, and then pipette to mix thoroughly. Incubate the mix at room temperature for **5 min**.

e. Set up PCR Library Mix according to Table 4-3 to start the amplification process of fragmented cDNA.

Table 4-3 PCR Library Mix

Components	1X (μL)
Fragmentation product	25
PCR Barcode Primer Mix	25
PCR Amplification Mix	50
Total	100

Please refer to Appendix A for guidelines for using PCR Barcode Primer Mix.

f. Vortex and spin down briefly the reaction mix prepared above. Incubate it in a thermocycler with the following incubation protocol (Table 4-4) and start the program.

Table 4-4 PCR Amplification Program (for 100μL)

Temperature	Time	Cycle
(Heated lid) 105 °C	on	-
95°C	5 min	1
98°C	20 sec	13
58°C	20 sec	
72°C	30 sec	
72°C	5 min	1
12°C	Hold	-

g. Take 1 μL of the PCR product and use the Qubit dsDNA HS Kit to measure the concentration. The concentration is usually around 10-100 ng/μL.

4.3. PCR Product Size Selection

a. Mix the PCR product obtained above with the magnetic beads in a volume ratio of 1 : 0.55 (PCR product : beads = 100 μL : 55 μL) in a PCR tube. Vortex the mixture then incubate it at room temperature for **5 min**.

b. Short spin the reaction mix and place the tube onto a magnetic separation rack for **3 min** until it becomes clear. Then, carefully transfer the supernatant to a new PCR tube.

Keep the supernatant and discard the beads.

c. Add **15 μL** of beads to the new PCR tube with the supernatant from step b. Vortex to mix thoroughly. Incubate at room temperature for **5 min**.

d. Spin down and place the tube onto a magnetic separation rack for **3-5 min** until it becomes clear. Carefully discard the supernatant with a pipette.

e. Keep the tube on the magnetic separation rack and add **200 μL** of freshly prepared 80% ethanol to wash the beads by rotating the tube on the magnetic rack. Incubate for **30 sec** and carefully remove the supernatant with a pipette.

f. Repeat step e. one more time.

g. Spin-down the tube and put it on the magnetic rack to extract the beads out of the liquid. Use a smaller pipette tip to remove the remaining liquid and discard it.

h. Air-dry the beads for **3-5 min** until the bead surface is not reflective or cracked.

i. Mix the dried beads with **20 μL** of TE buffer, vortex to mix and incubate at room temperature for **5 min**. Spin down briefly and place the centrifuge tube onto a magnetic separation rack for **3 min** until the liquid becomes clear. Transfer the supernatant to a new **1.5 mL** tube.

Keep the supernatant.

j. Take **1 μL** of purified PCR product and measure the concentration with Qubit dsDNA HS Kit. Use Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer(TM) (Advanced Analytical) or other equipment based on the principle of electrophoretic separation to detect the fragment distribution of the purified PCR products. The main peak of fragment distribution is required to be 200-600 bp (Figure 4). Normally the PCR yield is higher than 100 ng. QC

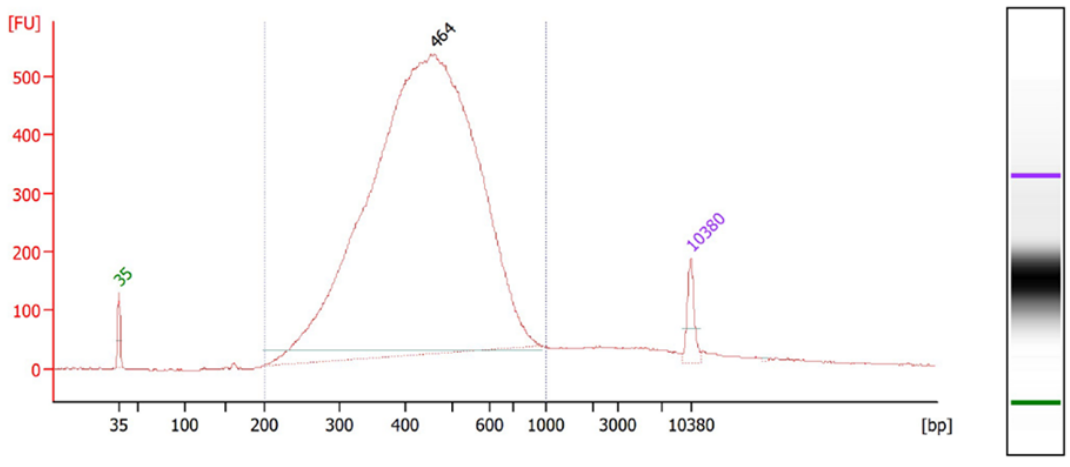
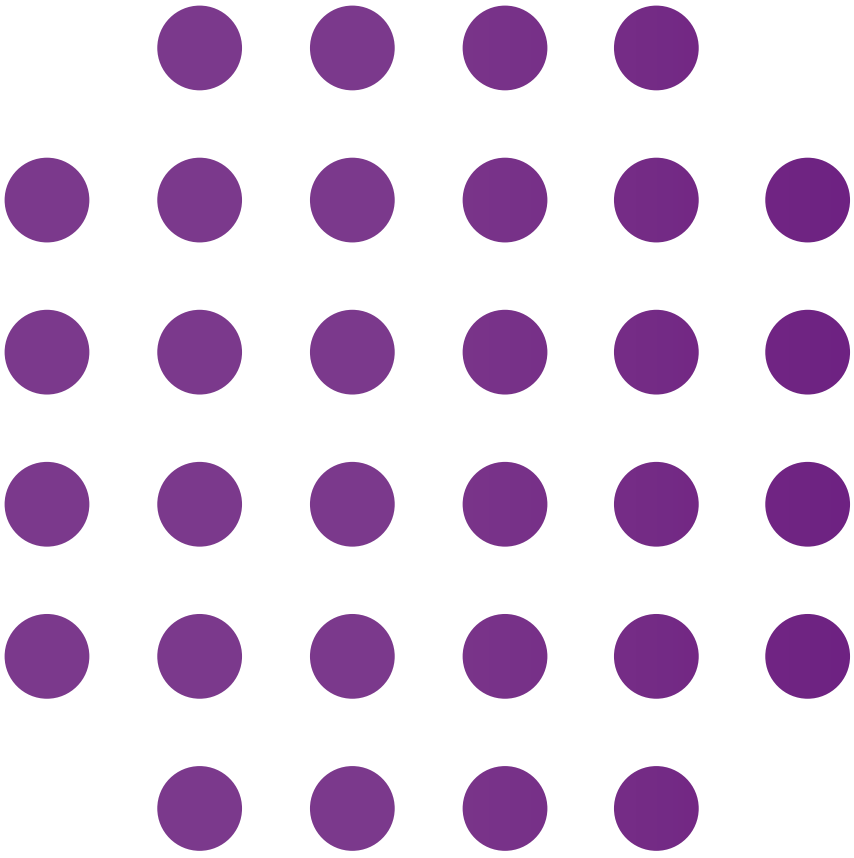


Figure 2. Agilent 2100 Bioanalyzer fragment size distribution of the purified PCR product

CHAPTER 5
LIBRARY CONSTRUCT & SEQUENCING



This chapter introduces the compatible sequencing instruments and sequencing reagents for the Stereo-seq system. The library construct is illustrated in Figure 4.

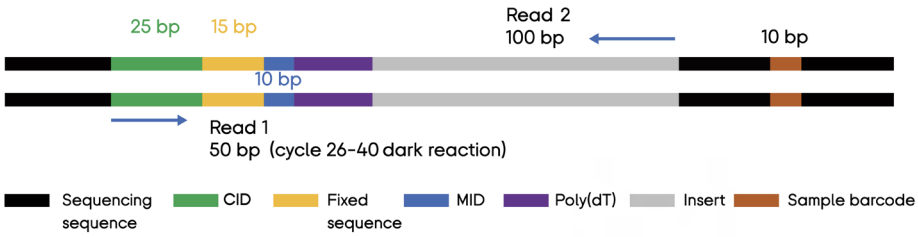


Figure 3. Stereo-seq Transcriptome Library Construct

Please refer to the user manual of High-throughput Sequencing Primer Kit (STOmics), Cat. no. 940-000037-00 for DNB preparation.

Use the following parameters to perform the sequencing run:

- Without sample barcode sequenced (for only one sample): choose paired-ended mode with 50 cycles of Read 1 and 100 cycles of Read 2. Use dark cycles on Read 1 from 26 to 40 cycles.
- With sample barcode sequenced (for two or more samples): choose paired-ended mode with 50 cycles of Read 1 and 100 cycles of Read 2 and an additional 10 cycles of sample barcode. Use dark cycles on Read 1 from 26 to 40 cycles.

Please read the corresponding user manual High-throughput Sequencing Primer Kit (STOmics), Cat. no. 940-000037-00 carefully before performing sequencing and strictly follow the instructions. If you have any questions about sequencing, please contact your local MGI account manager or technical support.

Appendix A: PCR Barcode Primer Mix Use Rules

The PCR Barcode Primer Mix in this kit is a pre-mixed barcode combination with a balanced set of bases, which can be randomly selected for use by the customer. Splitting barcode is required in sequencing for two or more samples in the same lane (in order to distinguish your sequencing samples). If there is only one sample, do not split barcode for sequencing. The following table is the barcode sequence number in each pre-set PCR Barcode Primer Mix.

PCR Barcode Primer Mix Name	Contains the Barcode Sequence Number							
PCR Barcode Primer Mix (Barcode 57~64)	57	58	59	60	61	62	63	64
PCR Barcode Primer Mix (Barcode 81~88)	81	82	83	84	85	86	87	88
PCR Barcode Primer Mix (Barcode 89~96)	89	90	91	92	93	94	95	96
PCR Barcode Primer Mix (Barcode 97~104)	97	98	99	100	101	102	103	104